Journal of Plant Physiology xxx (2011) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

# Reciprocal regulation between *AtNRT2.1* and *AtAMT1.1* expression and the kinetics of $NH_4^+$ and $NO_3^-$ influxes

Gemma Camañes[a,1], Elena Bellmunt[a,1], Javier García-Andrade[b], Pilar García-Agustín[a], Miguel Cerezo[a,*]

[a] *Área de Fisiología Vegetal, Departamento de Ciencias Agrarias y del Medio Natural, Escuela Superior de Tecnología y Ciencias Experimentales, Universitat Jaume I, E-12071 Castellón de la Plana, Spain*
[b] *Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia – Consejo Superior de Investigaciones Científicas, Ciudad Politécnica de la Innovación, 46022 Valencia, Spain*

ARTICLE INFO

*Article history:*
Received 5 May 2011
Received in revised form 17 October 2011
Accepted 18 October 2011

*Keywords:*
AMT1
NRT2
$NH_4^+$ influx
$NO_3^-$ influx

ABSTRACT

Our results show that *AtNRT2.1* expression has a positive effect on the $NH_4^+$ ion influx, mediated by the HATS, as also occurs with *AtAMT1.1* expression on the $NO_3^-$ ion influx. *AtNRT2.1* expression plays a key role in the regulation of *AtAMT1.1* expression and in the $NH_4^+$ ion influx, differentiating the nitrogen source, and particularly, the lack of it. Nitrogen starvation produces a compensatory effect by *AtAMT1.1* when there is an absence of the *AtNRT2.1* gene. Our results also show that, in the *atnrt2* mutant lacking both *AtNRT2.1* and *AtNRT2.2*, gene functions present different kinetic parameters on the $NH_4^+$ ion influx mediated by the HATS, according to the source and availability of nitrogen. Finally, the absence of *AMT1.1* also produces changes in the kinetic parameters of the $NO_3^-$ influx, showing different $V_{max}$ values depending on the source of nitrogen available.



## Introduction

Nitrogen (N) can be found in the soil as a complex blend of organic and inorganic compounds, with $NO_3^-$ and $NH_4^+$ ions as the main sources of mineral N available for plant nutrition. Because these N forms can show high variability in the soil, both in space and in time, plants have had to develop a set of different uptake systems to adapt themselves to the external conditions and their nutritional requirements. There are multigenic families, both for the $NO_3^-$ transporters and for the $NH_4^+$ transporters, indicating that different genes play different roles in N nutrition and allow the plant to adapt to different environmental conditions by means of regulating these genes. Members of the *NRT2* gene family encode high-affinity $NO_3^-$ transporters (Forde, 2000), whereas the *AMT1* multigenic family genes encode high-affinity $NH_4^+$ transporters in different plant species (Howitt and Udvardi, 2000).

Among the components of the *NRT2* family, up to 7 in *Arabidopsis thaliana* (Orsel et al., 2002), it seems that *NRT2.1* is a major component of the $NO_3^-$ HATS, as illustrated by the fact that several mutants disrupted the *NRT2.1* gene (Little et al., 2005; Li et al., 2007) or both *NRT2.1* – *NRT2.2* genes (Cerezo et al., 2001; Filleur et al., 2001) lost up to 72% of the high affinity $NO_3^-$ uptake activity. *NRT2.1* expression is regulated by N sources and their concentrations (Filleur and Daniel-Vedele, 1999; Gansel et al., 2001; Girin et al., 2007; Okamoto et al., 2003; Zhuo et al., 1999) and there is increasing evidence that NRT2 transporters are also regulated at the posttranscriptional level in various species (Fraisier et al., 2000; Wirth et al., 2007). Castaings et al. (2011) suggest that $NO_3^-$ is rapidly and specifically sensed by plant cells and that a $NO_3^-$ signaling pathway adjusts the expression of a large set of genes to adapt cell and organ metabolism and growth to N availability.

With regard to the *AMT1* family, the *AtAMT1.1*, *AtAMT1.2* and *AtAMT1.3* genes contribute to the influx mediated by the HATS. Influx measurements in a T-DNA insertion line showed that AMT1.1 is responsible for about 30% of the ammonium acquisition in *Arabidopsis* roots (Kaiser et al., 2002). It appears that the *AtAMT1.1* gene may be responding to differences in N concentration and source. *AMT1.1* expression is regulated similarly to *NRT2.1* by N sources and their concentrations (Gazzarrini et al., 1999; Rawat et al., 1999; Camañes et al., 2009), and at the posttranslational level, AMT1.1 can be inactivated by C-terminal phosphorylation that interacts with neighboring subunits in a trimer (Loqué et al., 2007).

We can draw from all of these studies that the regulation of N uptake is a complex and highly regulated process that depends on both endogenous and exogenous factors of the plant. Recently, we have advanced in molecular knowledge of N sensing in plants, such as the dual function of the nitrate transport CHL1 (Muños et al., 2004; Ho et al., 2009; Wang et al., 2009), the roles of different transcription factors (Rubin et al., 2009; Wang et al., 2009) or of the protein kinases (Ho et al., 2009; Hu et al., 2009). Proof

* Corresponding author.
*E-mail address:* cerezo@camn.uji.es (M. Cerezo).
[1] These authors contributed equally to this work.

0176-1617/$ – see front matter © 2011 Elsevier GmbH. All rights reserved.
doi:10.1016/j.jplph.2011.10.011

of the complexity of the process can be obtained when observing interactions among different genes. In *A. thaliana*, the reciprocal regulating interaction that might exist in the expression of genes from the same family, and even from different families, has been shown (Cerezo et al., 2001; Muños et al., 2004). Krouk et al. (2006) showed that *NRT1.1* expression takes part in *NRT2.1* expression. Kaiser et al. (2002) showed the existence of a compensatory effect by the *AtAMT1.2* and *AtAMT1.3* genes when there is an absence of the *AtAMT1.1* gene. This compensatory effect also occurs with the *AtNRT2.2* gene. This gene makes only a small contribution to the $NO_3^-$ HATS, except that when *AtNRT2.1* is lost, its contribution increases and results in a partial compensation (Li et al., 2007). NAR2.1/NRT3.1 has been described as a trans-membrane protein that is involved in the NRT2.1 transporter post-translational regulation, but it has no transporter activity in itself (Okamoto et al., 2006; Orsel et al., 2006). Surprisingly, *atnar2.1* null mutants display stronger HATS activity reduction than the double mutant *atnrt2.1-atnrt2.2* (Orsel et al., 2006). There are some indications that NRT2.1 could also act either as a $NO_3^-$ sensor or signal transducer to coordinate the development of the root system and coordinate it with nutritional cues (Little et al., 2005). Because NRT2.1 presents a dual nutrient transport/signaling function, it could be considered a putative $NO_3^-$ transceptor (Gojon et al., 2011).

Due to the importance of the *NRT2.1* and *AMT1.1* genes in N nutrition in low external N concentration conditions, and due to the fact that both genes respond by de-repressing with a lack of N and induction because of low N concentrations, the aim of this work was to examine the effect of *AtNRT2.1* expression on the regulation of *AtAMT1.1* expression and on the kinetic parameters of the influx, and *vice versa*.

## Materials and methods

### *Plant material and growth conditions*

The *Arabidopsis thaliana* genotypes used in this study were the wild type Wassilewskija (Ws) the Ws mutant *atnrt2* (*atnrt2.1-2.2*) (Filleur et al., 2001), the EMS *lin1* line (Little et al., 2005) and the *amt1-1:T-DNA* mutant (Kaiser et al., 2002) and their corresponding wild type in the Col-0 and Col-3 gl1 backgrounds, respectively. All plant genotypes were grown hydroponically as described in Lejay et al. (1999). The seeds were germinated directly on top of modified eppendorf tubes filled with pre-wetted sand. The tubes were then positioned on floating rafts transferred on tap water in a growth chamber under the following environmental conditions: light/dark cycle 8 h/16 h, light intensity 200 μmol $s^{-1}$ $m^{-2}$, temperature 25 °C/20 °C, hygrometry 80%. After 1 week, the tap water was replaced by complete nutrient solution. Then, plants were grown until the age of 6 weeks on a 1 mM $NH_4NO_3$ nutrient solution (control plants), which prevented any growth difference between the four genotypes (data not shown) (Lejay et al., 1999; Cerezo et al., 2001). Before the experiments, nitrogen was supplied as 1 mM $NO_3^-$ for 24 h (N-$NO_3^-$ induced plants), 0.1 mM $NH_4^+$ 24 h (N-$NH_4^+$ induced plants) or 48 h on nitrogen-free solution (de-repressed plants). The other nutrients were added as described in Lejay et al. (1999). Nutrient solutions were aerated vigorously, renewed weekly and the day before the experiments, pH was adjusted at 6.0.

All experiments were repeated three or five times, and typical results are shown.

### *Root influxes of $^{15}NO_3^-$ and $^{15}NH_4^+$*

An influx of either $^{15}NO_3^-$ (Col and *amt1-1:T-DNA* plants) or $^{15}NH_4^+$ (WS and *atnrt2* plants) into the roots was assayed as described by Delhon et al. (1995) and Gazzarrini et al. (1999), respectively. Control plants, N-$NO_3^-$ or N-$NH_4^+$ induced plants and de-repressed plants of four genotypes were sequentially transferred to 0.1 mM $CaSO_4$ for 1 min and to complete nutrient solution (pH 6.0) containing either $^{15}NO_3^-$ or $^{15}NH_4^+$ (98% atom excess $^{15}N$) for 5 min, at the concentrations indicated in figures. At the end of the $^{15}N$ labeling, roots were washed for 1 min in 0.1 mM $CaSO_4$ and were separated from shoots. The roots were dried at 70 °C for 48 h, weighed, crushed in a hammer-mill and analyzed for total $^{15}N$ content using an integrated system for continuous flow isotope ratio mass spectrometry (Euro-EA elemental analyser, EuroVector S.P.A. and Isoprime mass spectrometer, GV Instruments). Root influx is expressed in μmol $^{15}NO_3^-$ or $^{15}NH_4^+$ (g root DW)$^{-1}$ $h^{-1}$.

### *Kinetics of $^{15}NO_3^-$ and $^{15}NH_4^+$ influx*

The kinetics of $^{15}NO_3^-$ influx (Col and *amt1-1:T-DNA* plants) and $^{15}NH_4^+$ influx (WS and *atnrt2* plants) as a function of external $^{15}NO_3^-$ or $^{15}NH_4^+$ concentrations ($[^{15}NO_3^-]_0$ and $[^{15}NH_4^+]_0$) were measured with $[^{15}NO_3^-]_0$ or $[^{15}NH_4^+]_0$ ranging from 0.02 mM to 0.8 mM. For the kinetics studies, control plants, N-$NO_3^-$ or N-$NH_4^+$ induced plants and de-repressed plants of four genotypes were used. A data transformation method based on the Michaelis–Menten formalism was used to obtain $V_{max}$ and $K_m$ estimates. The experiment was repeated three times.

### *RNA extraction and Real-time PCR analysis*

Gene expression by quantitative real-time RT-PCR was performed using RNA samples extracted from root tissue using the Total Quick RNA kit (TALENT, Italy) according to the manufacturer's instructions. To avoid contaminating DNA, the samples were treated with DNAse I. A total of 1 μg of total RNA was annealed to oligo-dT and reverse transcribed using Omniscript Reverse Transcription kit (QIAGEN) to obtain cDNA. The sequences of the gene-specific oligonucleotides designed and used for real-time PCR are the following: *AMT1.1* forward: 5′acactgtggccagttaggcg3′ and reverse: 5′ccgtggggatgtctttgaga3′, *NRT2.1* forward: 5′agtcgcttgcacgttacctg3′ and reverse: 5′accctctgacttggcgttctc3′; *β-Tubuline* (*TUB*) forward: 5′cgattccgttctcgatgttgt3′ and reverse: 5′aatgagtgacacacttggaatcctt3′ and EF1α forward: 5′gtcgattctggaaagtcgacc3′ and reverse: 5′aatgtcaatggtgataccacgc3′. Real-time PCR was conducted using the QuantiTect™ SYBR Green PCR Kit (QIAGEN) and the SmartCycler II instrument (Cepheid). The experiment was repeated three times.

## Results and discussion

### *Is there reciprocal regulation between AtNRT2.1 and AtAMT1.1 expression and influxes, mediated by the HATS, of $NH_4^+$ and $NO_3^-$, respectively?*

The induction of $NH_4^+$ and $NO_3^-$ influxes mediated by the HATS and the induction of the *AtAMT1.1* and *AtNRT2.1* expression, by $NO_3^-$ and $NH_4^+$, together with de-repression due to a lack of N, has been shown previously (Filleur and Daniel-Vedele, 1999; Gazzarrini et al., 1999; Gansel et al., 2001; Okamoto et al., 2003). However, there are no studies examining the $NH_4^+$ influx and *AtAMT1.1* expression in plants which lack *AtNRT2.1* function as well as the influence of *AtAMT1.1* in $NO_3^-$ influx and *AtNRT2.1* expression, both genes involved in N uptake.

Our results show that when WS and *atnrt2* plants grow in 1 mM of $NH_4NO_3$ there are no significant differences in the $NH_4^+$ ion influx mediated by the HATS, and the average value is up to 60.5 ± 2.0 μmol (g DW)$^{-1}$ $h^{-1}$ (Fig. 1A). Under the same conditions, the $NO_3^-$ ion influx, mediated by the HATS, in Col and

Please cite this article in press as: Camañes G, et al. Reciprocal regulation between *AtNRT2.1* and *AtAMT1.1* expression and the kinetics of $NH_4^+$ and $NO_3^-$ influxes. J Plant Physiol (2011), doi:10.1016/j.jplph.2011.10.011

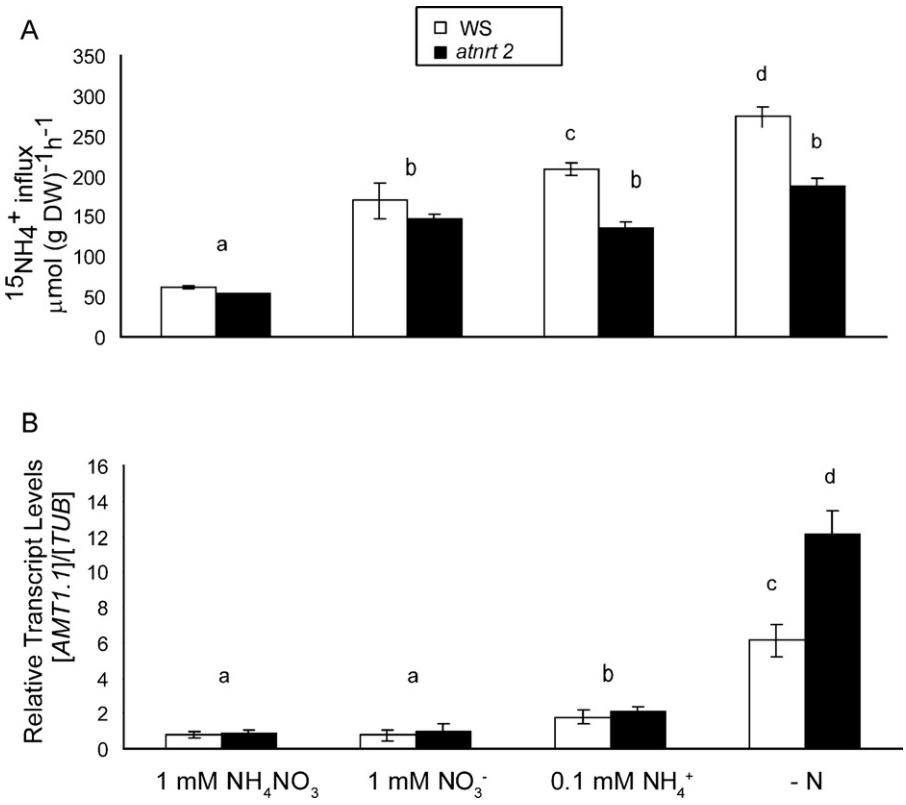


**Fig. 1.** Correlation between $^{15}NH_4^+$ influx and *AMT1.1* expression in WS and *atnrt2* after transfer of hydroponically grown plants from 1 mM $NH_4NO_3$ to 1 mM $NH_4NO_3$, 1 mM $NO_3^-$ or to 0.1 mM $NH_4^+$ during 24 h or to nitrogen deficiency during 48 h. *Arabidopsis* were grown hydroponically with N supplied as 1 mM $NH_4NO_3$ during 6 weeks. (A) Root $^{15}NH_4^+$ influx was assayed by 5-min labeling at 0.2 mM external $^{15}NH_4^+$ to determine the uptake activity of the HATS in WS and *atnrt2* plants. Each value is the mean of 15–20 replicates ± SE. (B) Real-time PCR analysis of the expression of *AMT1.1*. The plants were from the same experiment as those used for measurements of influx activity in (A). The *AMT1.1* transcript levels were normalized to the expression of *TUB* measured in the same samples. The experiment was repeated using *EF1α* with similar results. Each bar represents average data with standard error bars from three independent experiments obtained with a pool of 12 plants per point. Different letters on top of the bars indicate statistically significant differences ($P < 0.05$; *t* test).

*amt1-1*:*T-DNA* plants does not show significant differences and the average value is 18.3 ± 1.2 μmol (g DW)$^{-1}$ h$^{-1}$ (Fig. 2A). These influx values are similar to the order of magnitude observed by other authors in plants grown under similar conditions (Cerezo et al., 2001; Gansel et al., 2001; Loqué et al., 2006). Similarly, we observed that *AtAMT1.1* expression is equal in WS and *atnrt2* plants and *AtNRT2.1* expression does not show significant differences in Col and a*mt1-1*:*T-DNA* plants in these conditions. In addition, the level of expression of *AtAMT1.1* and *AtNRT2.1* are the same among the 4 genotypes (Figs. 1B and 2B). Although the exclusive nutrition with $NH_4^+$ may result in toxicity for plants (Britto and Kronzucker, 2002), the fact that plants prefer to absorb $NH_4^+$ rather than $NO_3^-$ when both forms of N are present in the environment at a low concentration has been shown in *A. thaliana* and other vegetable species (Gebler et al., 1998; Gazzarrini et al., 1999; Camañes et al., 2009). This can be explained by the lower energy cost for the plant when assimilating N-$NH_4^+$ (Bloom et al., 1992). Although *AtAMT1.1* and *AtNRT2.1* expression are not different, the $NH_4^+$ ion influx is three times higher than the $NO_3^-$ ion influx, which could be explained by the larger capacity of HATS for $NH_4^+$ ion than for $NO_3^-$ ion (Kronzucker et al., 1995, 1996).

When WS and *atnrt2* plants are treated with 1 mM of $NO_3^-$ over 24 h, an induction of the $NH_4^+$ ion influx three times higher is observed compared to control plants (1 mM $NH_4NO_3$), with no differences between the two genotypes, and the average value is 170.7 ± 7.6 μmol (g DW)$^{-1}$ h$^{-1}$. This induction cannot be explained by an increase in *AtAMT1.1* expression, which remains constant and at the same level as in control plants (Fig. 1). Similarly, Col and *amt1.1*:*T-DNA* show an induction of the $NO_3^-$ ion influx of 41 ± 2.3 μmol (g DW)$^{-1}$ h$^{-1}$ and 28 ± 1.8 μmol (g DW)$^{-1}$ h$^{-1}$, respectively. This difference in the $NO_3^-$ influx value is significant and is not correlated to *AtNRT2.1* expression, which remains constant in both genotypes, although it shows an induction with an expression two times higher than in plants grown with 1 mM $NH_4NO_3$ (Fig. 2). Our results seem to indicate that *AtNRT2.1* expression does not exert any control on the $NH_4^+$ ion influx or on *AtAMT1.1* expression in the induction mediated by $NO_3^-$, whereas *AtAMT1.1* expression seems to be involved in the $NO_3^-$ ion influx induction without affecting the *AtNRT2.1* expression.

When plants are treated with 0.1 mM $NH_4^+$ over 24 h, the $NH_4^+$ ion influx induction is produced both in WS and *atnrt2* plants, although the influx is almost 4-fold [220.0 ± 5.7 μmol (g DW)$^{-1}$ h$^{-1}$] higher in WS and 3-fold [150.3 ± 4.8 μmol (g DW)$^{-1}$ h$^{-1}$] higher in *atnrt2* compared to plants grown with 1 mM $NH_4NO_3$ (Fig. 1A). An induction of *AtAMT1.1* expression is also observed without differences between the two genotypes (Fig. 1B). Similarly, differing induction of $NO_3^-$ influx is produced in Col [58.6 ± 8.3 μmol (g DW)$^{-1}$ h$^{-1}$] and [42.0 ± 2.2 μmol (g DW)$^{-1}$ h$^{-1}$] in *amt1-1*:*T-DNA* plants (Fig. 2A). A very strong induction of *AtNRT2.1* expression is also produced and does not show differences between genotypes (Fig. 2B). These results seem to indicate that, in the induction mediated by $NH_4^+$, *AtNRT2.1* expression exerts some control over the $NH_4^+$ ion influx and none over *AtAMT1.1* expression. Under the same conditions, *AtAMT1.1* expression seems to be involved in the induction of the $NO_3^-$ ion influx without affecting *AtNRT2.1* expression.

When plants are under conditions lacking N for 48 h, significant differences are observed in both the $NH_4^+$ ion influx and

Please cite this article in press as: Camañes G, et al. Reciprocal regulation between *AtNRT2.1* and *AtAMT1.1* expression and the kinetics of $NH_4^+$ and $NO_3^-$ influxes. J Plant Physiol (2011), doi:10.1016/j.jplph.2011.10.011

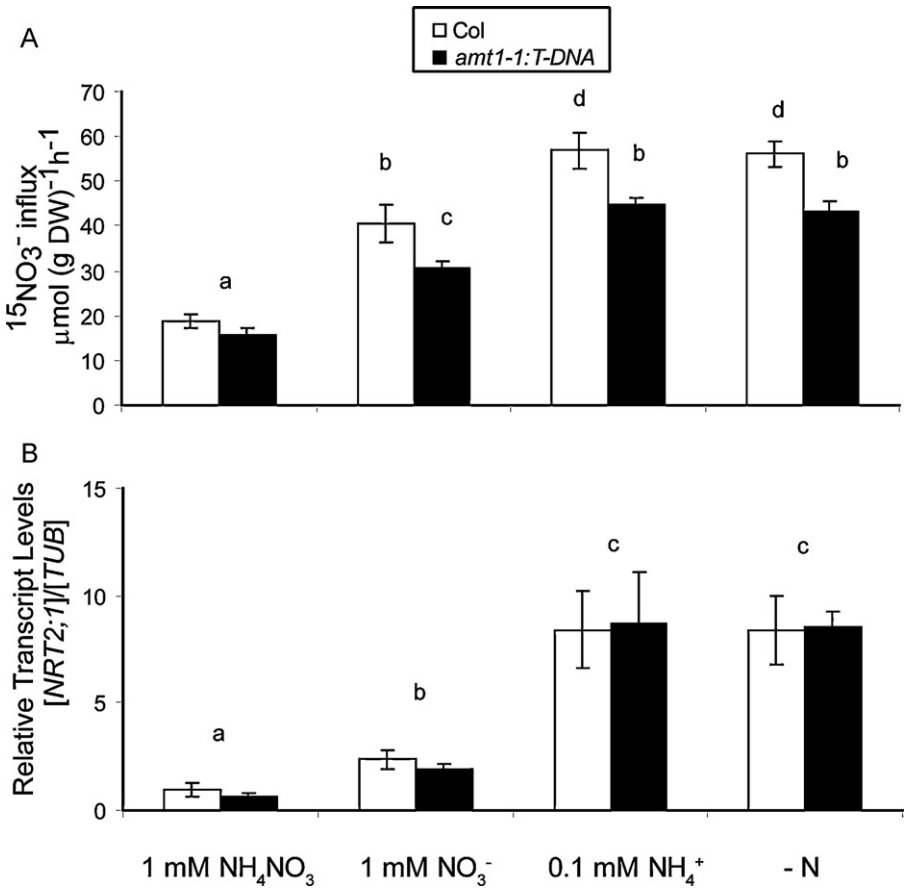


**Fig. 2.** Correlation between $^{15}NO_3^-$ influx and *NRT2.1* expression in Col and *amt1-1:T-DNA* after transfer of hydroponically grown plants from 1 mM $NH_4NO_3$ to 1 mM $NH_4NO_3$, 1 mM $NO_3^-$ or to 0.1 mM $NH_4^+$ during 24 h or to nitrogen deficiency during 48 h. *Arabidopsis* were grown hydroponically with N supplied as 1 mM $NH_4NO_3$ during 6 weeks. (A) Root $^{15}NO_3^-$ influx was assayed by 5-min labeling at 0.2 mM external $^{15}NO_3^-$ to determine the uptake activity of the HATS in Col and *Amt1-1:T-DNA* plants. Each value is the mean of 15–20 replicates ± SE. (B) Real-time PCR analysis of the expression of *NRT2.1*. The plants were from the same experiment as those used for measurements of influx activity in (A). The *NRT2.1* transcript levels were normalized to the expression of *TUB* measured in the same samples. The experiment was repeated using *EF1α* with similar results. Each bar represents average data with standard error bars from three independent experiments obtained with a pool of 12 plants per point. Different letters on top of the bars indicate statistically significant differences ($P < 0.05$; *t* test).

*AtAMT1.1* expression in WS and *atnrt2* plants (Fig. 1). The $NH_4^+$ ion influx is 275.1 ± 6.7 µmol (g DW)$^{-1}$ h$^{-1}$ in WS and 172.4 ± 4.4 µmol (g DW)$^{-1}$ h$^{-1}$ in *atnrt2* plants, and *AtAMT1.1* expression shows a great de-repression (approximately 6-fold more than in plants that have remained with 1 mM $NH_4NO_3$) in WS plants, whereas the *AtAMT1.1* gene de-repression in *atnrt2* plants is 12-fold (Fig. 1). A differential increase of the $NO_3^-$ influx around 60.3 ± 3.3 µmol (g DW)$^{-1}$ h$^{-1}$ and 40.2 ± 3.2 µmol (g DW)$^{-1}$ h$^{-1}$ also occurs in the Col and *amt1-1:T-DNA* plants respectively. This increment is similar to the one produced by the 0.1 mM of $NH_4^+$ treatment (Fig. 2A). N starvation de-repressed *AtNRT2.1* with no difference between both genotypes, and somehow this effect is also observed in $NH_4^+$ treatment (Fig. 2B). The results seem to indicate that, in de-repression mediated by the lack of N, *AtNRT2.1* expression exerts some control over the $NH_4^+$ ion influx and *AtAMT1.1* expression, whereas *AtAMT1.1* expression seems to be involved in the $NO_3^-$ ion influx induction without affecting *AtNRT2.1* expression.

Li et al. (2007) examined HATS activity in *AtNRT2.1* and *AtNRT2.2* single and double mutants and showed that *AtNRT2.1* is responsible for 72% of the HATS activity. This result suggests that *AtNRT2.2* makes only a small contribution to the system, except when *AtNRT2.1* is lost. In that case, its contribution increases and results in a partial compensation. The single mutant *AtNRT2.1* (*lin1*) was also subjected to N starvation for 48 h and we observed the same results as for the double mutant *atnrt2*. In conditions lacking N, Col and *lin1* plants show a great *AMT1.1* gene de-repression. This de-repression in *lin1* plants was double that in the wild type (Fig. 3). This result could indicate that the *AtNRT2.2* gene does not play an important role in controlling *AtAMT1.1* expression.

The differences observed in both ecotypes between the transcription levels of the *AtAMT1.1* and *AtNRT2.1* genes and their

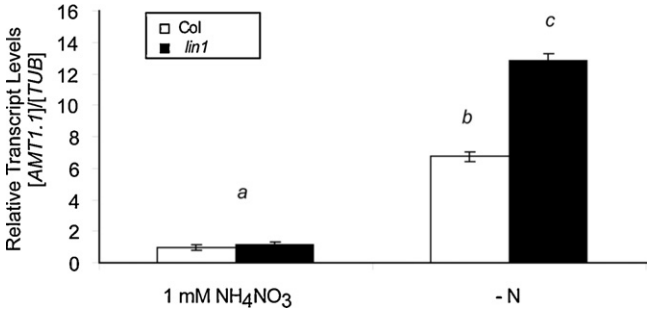


**Fig. 3.** *AMT1.1* expression in Col and *lin1* after transfer of hydroponically grown plants from 1 mM $NH_4NO_3$ to 1 mM $NH_4NO_3$ or to nitrogen deficiency during 48 h. *Arabidopsis* were grown hydroponically with N supplied as 1 mM $NH_4NO_3$ during 6 weeks. Real-time PCR analysis of the expression of *AMT1.1*. The *AMT1.1* transcript levels were normalized to the expression of *TUB* measured in the same samples. The experiment was repeated using *EF1α* with similar results. Each bar represents average data with standard error bars from three independent experiments obtained with a pool of 12 plants per point. Different letters on top of the bars indicate statistically significant differences ($P < 0.05$; *t* test).

Please cite this article in press as: Camañes G, et al. Reciprocal regulation between *AtNRT2.1* and *AtAMT1.1* expression and the kinetics of $NH_4^+$ and $NO_3^-$ influxes. J Plant Physiol (2011), doi:10.1016/j.jplph.2011.10.011

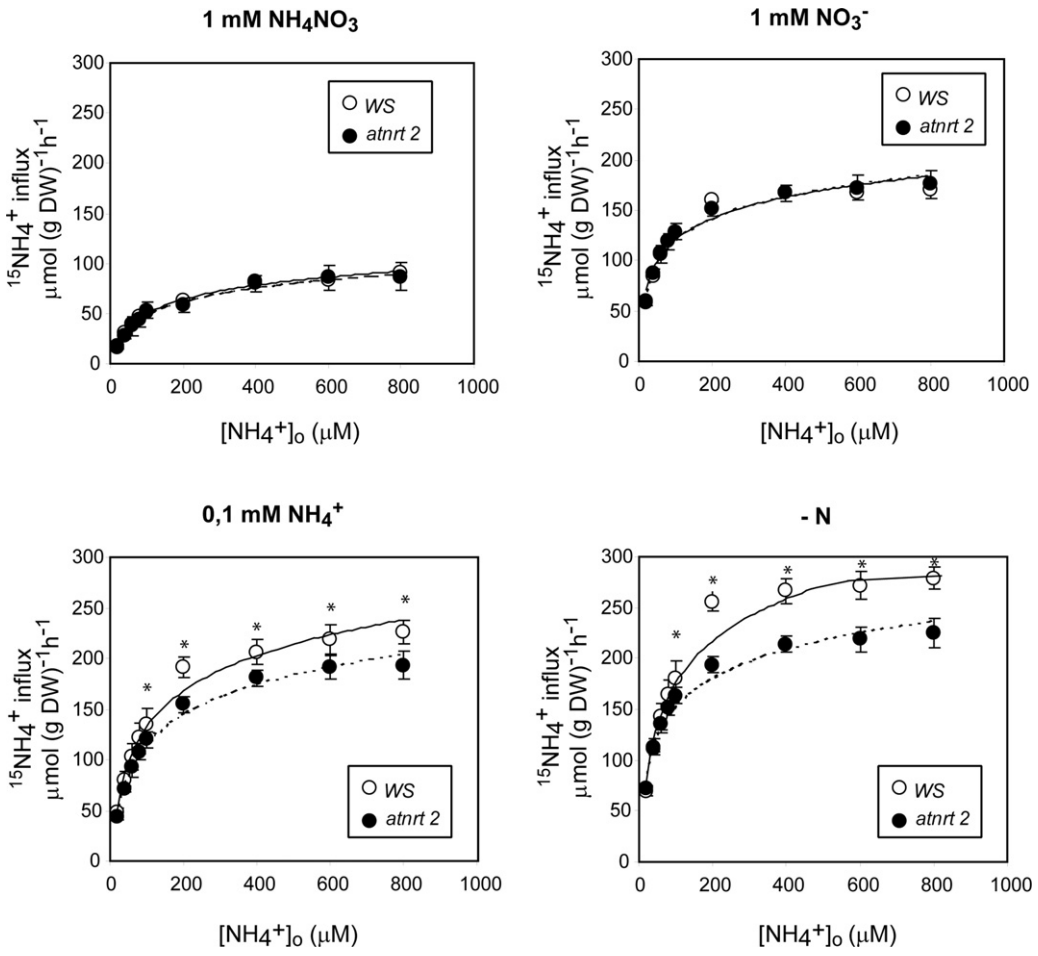


**Fig. 4.** Kinetics of $^{15}NH_4^+$ influx in *Arabidopsis* roots in the low $^{15}NH_4^+$ concentration range. *Arabidopsis* were grown hydroponically with N supplied as 1 mM $NH_4NO_3$ during 6 weeks. After that one group of plants were transferred to 1 mM $NH_4NO_3$, other group of plants were transferred to 1 mM $KNO_3$ and other group to 0.1 mM $(NH_4)_2SO_4$ during 24 h, and other group of plants were transferred to nitrogen-free nutrient solution (−N) during 48 h. $^{15}NH_4^+$ influx was measured at different concentrations of external $^{15}NH_4^+$. Each data is the mean of 30 replicates ± SE. *Statistically significant differences ($P < 0.05$; *t* test).

respective influxes of $NH_4^+$ and $NO_3^-$ (Figs. 1 and 2) led us to study the kinetic parameters in the different treatments. The kinetic parameters of the $NH_4^+$ ion influx mediated by HATS in WS and *atnrt2* plants and, $NO_3^-$ ion influx mediated also by the HATS in Col and *amt1-1:T-DNA* plants, were measured. Our results show that plants grow with a continuous contribution from both N sources (1 mM $NH_4NO_3$), and do not exhibit significant differences between the kinetic parameters of the WS and *atnrt2* plants (Fig. 4 and Table 1) or Col and *amt1-1:T-DNA* plants (Fig. 5 and Table 1). The kinetic parameters again show the greater capacity of the $NH_4^+$ ion influx mediated by the HATS when compared to that of $NO_3^-$ ion. The value obtained for $V_{max}$ is three times higher, with no significant differences in the $K_m$ (Table 1) when plants grow with both sources of N. These values are similar to those obtained by other researchers in analogous culture conditions (Cerezo et al., 2001; Gansel et al., 2001; Loqué et al., 2006). These results indicate that, in mineral nutrition conditions with both sources of N, there is no reciprocal effect of *AtNRT2.1* and *AtAMT1.1* expression on the $NH_4^+$ and $NO_3^-$ influxes, respectively, or over the expression of each gene.

A different response was observed among the two ecotypes in the induction treatment with 1 mM $NO_3^-$. We found a 2-fold induction compared to the control with no significant differences between genotypes (Fig. 4 and Table 1) in the capacity and affinity ($V_{max}$ and $K_m$) of the NH4+ influx, whereas an induction is also produced but with significant differences between Col and *amt1.1:T-DNA* plants. In this case, there is a loss of 15% in the induction capacity in mutant plants, and this effect happens

**Table 1**
Kinetic parameters for saturable of $^{15}NH_4^+$ influx (WS and *atnrt2* plants) or $^{15}NO_3^-$ influx (Col and *amt1-1:T-DNA*). *Arabidopsis* were grown hydroponically with N supplied as 1 mM $NH_4NO_3$ during 6 weeks. To kinetics studies control plants (1 mM $NH_4NO_3$), induced plants (1 mM $NO_3^-$), induced plants (0.1 mM $NH_4^+$) and de-repressed plants (−N) were used. $K_m$ is measured in µM and $V_{max}$ in [µmol (g DW)$^{-1}$ h$^{-1}$].

| | 1 mM $NH_4NO_3$ | | 1 mM $NO_3^-$ | | 0.1 mM $NH_4^+$ | | −N | |
|---|---|---|---|---|---|---|---|---|
| | $K_m$ | $V_{max}$ | $K_m$ | $V_{max}$ | $K_m$ | $V_{max}$ | $K_m$ | $V_{max}$ |
| *WS* | 95 ± 6 | 102 ± 10 | 40 ± 3 | 178 ± 12 | 85 ± 5 | 250 ± 15* | 57 ± 4 | 302 ± 15* |
| *atnrt2* | 100 ± 8 | 99 ± 8 | 44 ± 2 | 185 ± 15 | 82 ± 6 | 217 ± 12* | 52 ± 6 | 238 ± 12* |
| *Col* | 124 ± 12 | 32 ± 2 | 100 ± 9 | 58 ± 3* | 120 ± 9 | 76 ± 5* | 30 ± 2 | 81 ± 5* |
| *amt1.1:T-DNA* | 115 ± 9 | 28 ± 2 | 120 ± 11 | 49 ± 2* | 115 ± 11 | 62 ± 5* | 28 ± 2 | 60 ± 4* |

* Statistically significant differences between values ($P < 0.05$; *t* test).

Please cite this article in press as: Camañes G, et al. Reciprocal regulation between *AtNRT2.1* and *AtAMT1.1* expression and the kinetics of $NH_4^+$ and $NO_3^-$ influxes. J Plant Physiol (2011), doi:10.1016/j.jplph.2011.10.011

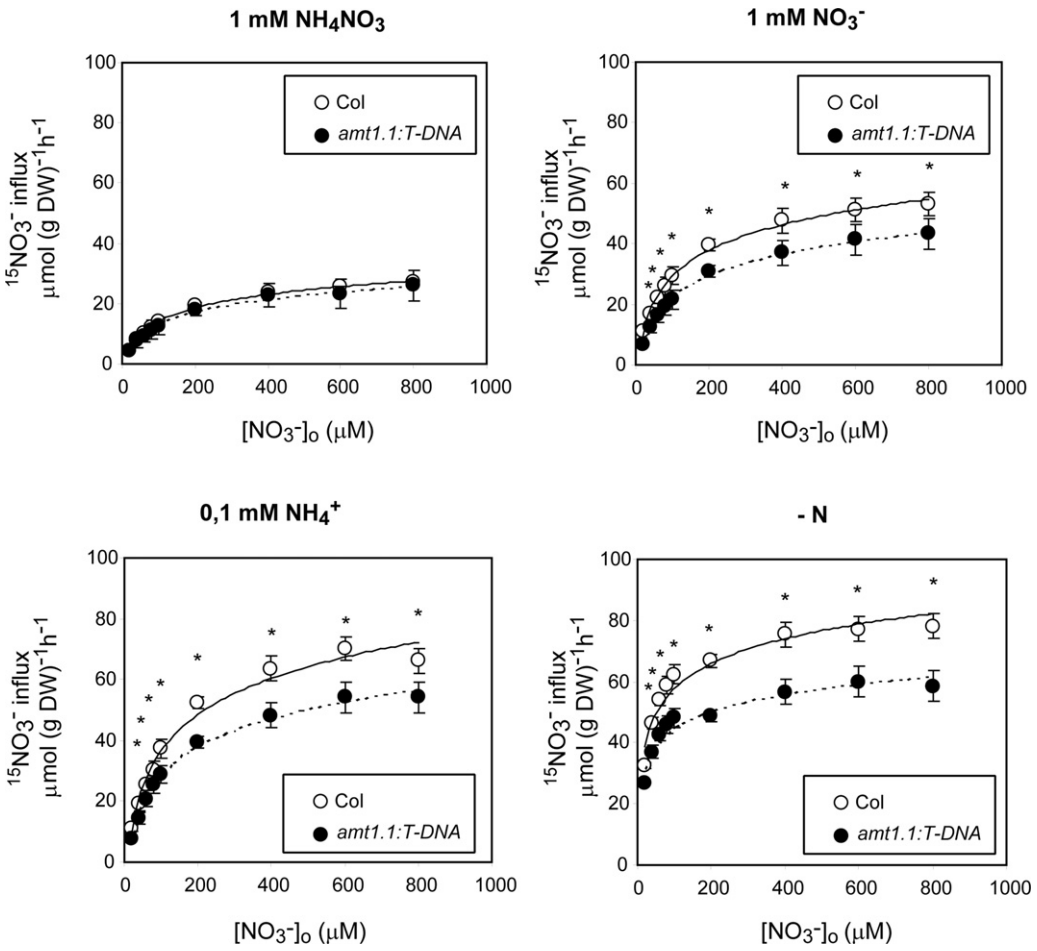


**Fig. 5.** Kinetics of $^{15}NO_3^-$ influx in *Arabidopsis* roots in the low $^{15}NO_3^-$ concentration range. *Arabidopsis* were grown hydroponically with N supplied as 1 mM $NH_4NO_3$ during 6 months. After that one group of plants were transferred to 1 mM $NH_4NO_3$, other group of plants were transferred to 1 mM $KNO_3$ and other group to 0.1 mM $(NH_4)_2SO_4$ during 24 h, and other group of plants were transferred to nitrogen-free nutrient solution (−N) during 48 h. $^{15}NO_3^-$ influx was measured at different concentrations of external $^{15}NO_3^-$. Each data is the mean of 30 replicates ± SE. *Statistically significant differences ($P < 0.05$; *t* test).

without modifying their affinity for the substrate (Fig. 5 and Table 1). These results suggest that *AtNRT2.1* expression induced by $NO_3^-$ does not have an influence on *AtAMT1.1* expression and $NH_4^+$ ion influx, but that *AtAMT1.1* is important for the regulation of $NO_3^-$ uptake.

When plants are treated with 0.1 mM $NH_4^+$, similar results are obtained in both ecotypes if compared with its corresponding mutant. In WS and *atnrt2* plants there is a higher influx induction than the produced in the previous treatment. Nevertheless, this difference in the induction can be more easily attributed to the increase of $V_{max}$ than to the decrease of $K_m$ (the affinity for the substrate is not altered in these plants) (Fig. 4 and Table 1). *atnrt2* mutant plants lost 13% of their response capacity to the induction with $NH_4^+$. Col and *amt1-1:T-DNA* plants also undergo a differential induction with this treatment in which $K_m$ is not altered, while $V_{max}$ is increased in both cases, although in *amt1-1:T-DNA* mutant plants there is a smaller increase, such as 18% (Fig. 5 and Table 1). It appears that *AtNRT2.1* expression does not have an effect on the induction of *AtAMT1.1* expression, but it somehow regulates the $NH_4^+$ ion influx, modifying the $V_{max}$ value. Likewise, *AtAMT1.1* expression does not affect the induction of *AtNRT2.1*, but it affects the $V_{max}$ value. The most notable results regarding the function of the *AtNRT2.1* and *AtAMT1.1* expression are those obtained with the treatment lacking N. In that case, there is no difference between the affinity for $NH_4^+$ ion in WS and *atnrt2* plants, which is higher than in plants that have grown with 1 mM $NH_4NO_3$ or in those plants treated with N-$NH_4^+$. Nevertheless, the capacity for the ion influx is diminished by 22% in *atnrt2* plants (Fig. 4 and Table 1). Moreover, a strong difference in *AtAMT1.1* expression exists between genotypes (Figs. 1 and 3). With regard to Col and *amt1-1:T-DNA* plants (Fig. 5 and Table 1), similar results to those obtained in the treatment with N-$NH_4^+$ are found; these similar results refer to $V_{max}$ (18% less in mutant plants), without altering $K_m$ between genotypes. It reaches the lowest values among the 4 treatments. It appears that *AtNRT2.1* expression affects the de-repression of the *AtAMT1.1* gene by means of some transcriptional mechanism, and it also acts on the regulation of $NH_4^+$ uptake. On the other hand, *AtAMT1.1* expression does not affect *AtNRT2.1* expression, but we observed a modification of the $V_{max}$ value.

For first time, we show the effect of a gene involved in N transport over another gene of a different family but involved in the N uptake. Taking these results together, it seems that the functions and importance of both *AtNRT2.1* and *AtAMT1.1* differ with respect to the regulation of one gene over the others, in different nutritional conditions in which the sources and concentrations of available N are altered. It is possible that *AtNRT2.1* expression may be a sign that could inform the plant of the type of N source available. Ho et al. (2009) confirmed the signaling function for other $NO_3^-$ transporters such as NRT1.1. It would also play an important role in regulating the *AtAMT1.1* expression at the transcriptional level. Its role as a long distance signal that indicates lack of N to the whole plant has been reported by Gansel et al. (2001). *AtAMT1.1*

Please cite this article in press as: Camañes G, et al. Reciprocal regulation between *AtNRT2.1* and *AtAMT1.1* expression and the kinetics of $NH_4^+$ and $NO_3^-$ influxes. J Plant Physiol (2011), doi:10.1016/j.jplph.2011.10.011

expression would never regulate *AtNRT2.1* expression at the transcriptional level with different N sources.

Taking all these results into consideration, *AtNRT2.1* expression seems to favor the $NH_4^+$ uptake, mediated by the HATS differently, depending on the N source or on the lack of this element. It plays a major role in the *AtAMT1.1* gene de-repression in conditions lacking N, showing differences between WS and *atnrt2* that could indicate a compensatory effect of *AtAMT1.1* expression due to the absence of *AtNRT2.1*. *AtAMT1.1* expression also favors the $NO_3^-$ ion influx by means of some mechanism independently of the N source used ($N\text{-}NH_4^+$ or $N\text{-}NO_3^-$). Moreover, it seems that it does not affect the regulation of the *AtNRT2.1* expression.

To explain the discrepancy observed between the *AtAMT1.1* and *AtNRT2.1* gene transcription levels and their respective $NH_4^+$ and $NO_3^-$ ion influx, it is possible that, in mutant plants, other AMT1 or NRT2 transporters could be affected, modifying the $NH_4^+$ and $NO_3^-$ HATS activity. Even proteins not belonging to any of these gene families, like NAR2/NRT3-1, could be altered on the *atamt1.1-TDNA* mutant, triggering a reduction of $NH_4^+$ uptake, or AtAMT1.1 and AtNRT2.1 transporters could be regulated at the post-transcriptional level under these conditions.

It is not surprising that *AtNRT2.1* expression plays a more complex role than the *AtAMT1.1*. Recently, *AtNRT2.1* was suggested as a putative $NO_3^-$ transceptor because it presents a dual nutrient transport/signaling function (Gojon et al., 2011). Remans et al. (2006) concluded that the NRT2.1 protein is responsible for the coordination of the root development with external availability of $NO_3^-$. We believe that *AtNRT2.1* expression represents a key point in producing the induction and/or de-repression of the *AtAMT1.1* gene, as well as the $NH_4^+$ ion influx regulation, mediated by the HATS, depending on the N source.

## Acknowledgements

We thank Dr. Françoise Daniel-Vedele (Unité de la Nutrition Azoté des Plantes, INRA, Versailles, France); Dr. Jocelyn Malamy (University of Chicago, EEUU) and Dr. Nicolaus von Wirén (Molekulare Pflanzenernährung, Universität Hohenheim, Stuttgart, Germany) for providing the *atnrt2* mutant, the *lin1* mutant and the *amt1-1:T-DNA* mutant, respectively. This work was supported by the Pla de Promoció de la Investigació de la Universitat Jaume I 2004 and 2010 (P1 1A2004-22 and P1.1B2010-06). We thank the Servicio Central de Instrumentación Científica (SCIC) of the Universitat Jaume I where $^{15}N$ analysis was performed.

## References

Bloom AJ, Sukrapanna SS, Warner RL. Root respiration associated with ammonium and nitrate absorption and assimilation by barley. Plant Physiol 1992;99:1294–301.
Britto DT, Kronzucker HJ. $NH_4^+$ toxicity in higher plants: a critical review. J Plant Physiol 2002;159:567–84.
Camañes G, Cerezo M, Primo-Millo E, Gojon A, García-Agustín P. Ammonium transport and *CitAMT1* expression are regulated by N in *Citrus* plants. Planta 2009;229:331–42.
Castaings L, Marchive C, Meyer C, Krapp A. Nitrogen signalling in *Arabidopsis*: how to get insights into a complex signalling network. J Exp Bot 2011;62:1391–7.
Cerezo M, Tillard P, Filleur S, Munos S, Daniel-Vedele F, Gojon A. Major alterations of the regulation of root $NO_3^-$ uptake are associated with the mutation of *Nrt2.1* and *Nrt2.2* genes in *Arabidopsis*. Plant Physiol 2001;127:262–71.
Delhon P, Gojon A, Tillard P, Passama L. Diurnal regulation of $NO_3^-$ uptake in soybean plants. 1. Changes in $NO_3^-$ influx, efflux, and N utilization in the plant during the day–night cycle. J Exp Bot 1995;46:1585–94.
Filleur S, Daniel-Vedele F. Expression analysis of a high-affinity nitrate transporter isolated from *Arabidopsis thaliana* by differential display. Planta 1999;207:461–9.
Filleur S, Dorbe MF, Cerezo M, Orsel M, Granier F, Gojon A, et al. An *Arabidopsis* T-DNA mutant affected in *Nrt2* genes is impaired in nitrate uptake. FEBS Lett 2001;489:220–4.
Forde BG. Nitrate transporters in plants: structure, function and regulation. Biochim Biophys Acta 2000;1465:219–35.
Fraisier V, Gojon A, Tillard P, Daniel-Vedele F. Constitutive expression of putative high-affinity nitrate transporter in *Nicotiana plumbaginifolia*: evidence for post-transcriptional regulation by a reduced nitrogen source. Plant J 2000;23:489–96.
Gansel X, Munos S, Tillard P, Gojon A. Differential regulation of the $NO_3^-$ and $NH_4^+$ transporter genes *AtNrt2.1* and *AtAmt1.1* in *Arabidopsis*: relation with long-distance and local controls by N status of the plant. Plant J 2001;26:143–55.
Gazzarrini S, Lejay T, Gojon A, Ninnemann O, Frommer WB, von Wirén N. Three functional transporters for constitutive, diurnally regulated, and starvation-induced uptake of ammonium into *Arabidopsis* roots. Plant Cell 1999;11:937–47.
Gebler A, Schneider S, von Sehgbusch D, Weber P, Hanemann U, Huber C, et al. Field and laboratory experiments on net uptake of nitrate and ammonium by the roots of spruce (*Picea abies*) and beech (*Fagus sylvatica*) trees. New Phytol 1998;138:275–85.
Girin T, Lejay L, Wirth J, Widiez T, Palenchar PM, Nazoa P, et al. Identification of a 150 bp cis-acting element of the AtNRT2.1 promoter involved in the regulation of gene expression by the N and C status of the plant. Plant Cell Environ 2007;30:1366–80.
Gojon A, Krouk G, Perrine-Walker F, Laugier E. Nitrate transceptor(s) in plants. J Exp Bot 2011;62:2299–308.
Ho CH, Lin SH, Hu HC, Tsay YF. CHL1 functions as a nitrate sensor in plants. Cell 2009;138:1184–94.
Howitt SM, Udvardi MK. Structure, function and regulation of ammonium transporters in plants. Biochim Biophys Acta 2000;1465:152–70.
Hu H, Wang Y, Tsay Y. AtCIPK8, a CBL-interacting protein kinase, regulates the low-affinity phase of the primary nitrate response. Plant J 2009;57:264–78.
Kaiser BN, Rawat SR, Siddiqi MY, Masle J, Glass ADM. Functional analysis of an *Arabidopsis* T-DNA "Knockout" of the high-affinity $NH_4^+$ transporter *AtAMT1.1*. Plant Physiol 2002;130:1263–75.
Kronzucker HJ, Siddiqi MY, Glass ADM. Kinetics of $NO_3^-$ influx in spruce. Plant Physiol 1995;109:319–26.
Kronzucker HJ, Siddiqi MY, Glass ADM. Kinetics of $NH_4^+$ influx in spruce. Plant Physiol 1996;110:773–9.
Krouk G, Tillard P, Gojon A. Regulation of the high-affinity $NO_3^-$ uptake system by NRT1.1-mediated $NO_3^-$ demand signalling in *Arabidopsis*. Plant Physiol 2006;142:1075–86.
Lejay L, Tillard P, Lepetit M, Olive FD, Filleur S, Daniel-Vedele F, et al. Molecular and functional regulation of two $NO_3^-$ uptake systems by N- and C-status of *Arabidopsis* plants. Plant J 1999;18:509–19.
Li W, Wang Y, Okamoto M, Crawford NM, Siddiqi MY, Glass ADM. Dissection of the *AtNRT2.1*, *AtNRT2.2* inducible high-affinity nitrate transporter gene cluster. Plant Physiol 2007;143:425–33.
Little DY, Rao H, Oliva S, Daniel-Vedele F, Krapp A, Malamy JE. The putative high-affinity nitrate transporter NRT2.1 represses lateral root initiation in response to nutritional cues. Proc Natl Acad Sci USA 2005;102:13693–8.
Loqué D, Lalonde S, Looger LL, von Wiren N, Frommer WB. A cytosolic trans-activation domain essential for ammonium uptake. Nature 2007;446:195–8.
Loqué D, Yuan L, Kojima S, Gojon A, Wirth J, Gazzarrini S, et al. Additive contribution of *AtAMT1.1* and *AtAMT1.3* to high-affinity ammonium uptake across the plasma membrane of nitrogen-deficient *Arabidopsis* roots. Plant J 2006;48:522–34.
Muños S, Cazettes C, Fizames C, Gaymard F, Tillard P, Lepetit M, et al. Transcript profiling in the *chl1-5* mutant of *Arabidopsis* reveals a role of the nitrate transporter *NRT1.1* in the regulation of another nitrate transporter, NRT2.1. Plant Cell 2004;16:2433–47.
Okamoto M, Kumar A, Li WB, Wang Y, Siddiqi MY, Crawford NM, et al. High-affinity nitrate transport in roots of *Arabidopsis* depends on expression of the NAR2-like gene *AtNrt3.1*. Plant Physiol 2006;140:1036–46.
Okamoto M, Vidmar JJ, Glass ADM. Regulation of *Nrt1* and *Nrt2* gene families of *Arabidopsis thaliana*: responses to nitrate provision. Plant Cell Physiol 2003;44:304–17.
Orsel M, Chopin F, Leleu O, Smith SJ, Krapp A, Daniel-Vedele F, et al. Characterization of a two-component high-affinity nitrate uptake system in *Arabidopsis*. Physiology and protein–protein interaction. Plant Physiol 2006;142:1304–17.
Orsel M, Krapp A, Daniel-Vedele F. Analysis of the NRT2 nitrate transporter family in *Arabidopsis*. Structure and gene expression. Plant Physiol 2002;129:886–96.
Rawat SR, Silim SN, Kronzucker HJ, Siddiqi MY, Glass ADM. *AtAmt1* gene expression and $NH_4^+$ uptake in roots of *Arabidopsis thaliana*: evidence for regulation by root glutamine levels. Plant J 1999;19:143–52.
Remans T, Nacry P, Pervent M, Girin T, Tillard P, Lepetit M, et al. A central role for the nitrate transporter NRT2.1 in the integrated morphological and physiological responses of the root system to nitrogen limitation in *Arabidopsis*. Plant Physiol 2006;140:909–21.
Rubin G, Tohge T, Matsuda F, Saito K, Scheible WR. Members of the LBD family of transcription factors repress anthocyanin synthesis and affect additional nitrogen responses in *Arabidopsis*. Plant Cell 2009;21:3567–84.
Wang R, Xing X, Wang Y, Tran A, Crawford NM. A genetic screen for nitrate regulatory mutants captures the nitrate transporter gene *NRT1.1*. Plant Physiol 2009;151:472–8.
Wirth J, Chopin F, Santoni V, Viennois G, Tillard P, Krapp A, et al. Regulation of root nitrate uptake at the NRT2.1 protein level in *Arabidopsis thaliana*. J Biol Chem 2007;282:23541–52.
Zhuo D, Okamoto M, Vidmar JJ, Glass ADM. Regulation of a putative high-affinity nitrate transporter (*AtNrt2.1*) in roots of *Arabidopsis thaliana*. Plant J 1999;17:563–8.

Please cite this article in press as: Camañes G, et al. Reciprocal regulation between *AtNRT2.1* and *AtAMT1.1* expression and the kinetics of $NH_4^+$ and $NO_3^-$ influxes. J Plant Physiol (2011), doi:10.1016/j.jplph.2011.10.011